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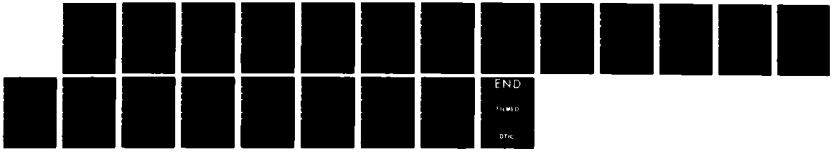

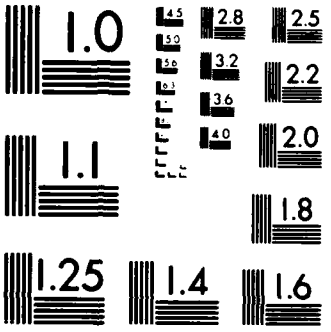

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Nephropathia epidemica (NE) virus, strain Puumala, was passaged successfully in C. gaperi voles. In addition to lung suspensions, urine of the infected voles was also shown to be infectious. Attempts to adapt the virus to cell cultures remained unsuccessful.

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Propagation and Characterization of the Etiologic Agent of Nephropathia Epidemica

Final Report

Carl-Henrik von Bonsdorff

June 1983

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SUMMARY

This is the final report for Grant No. DAMD17-82-G-9508. The work reported here has been done by Dr. Carl-Henrik von Bonsdorff, University of Helsinki, in collaboration with Dr. Joel M. Dalrymple, Department of Viral Biology, Virology Division, U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID), Fort Detrick, Maryland, from April to September 1982.

In this collaborative study between the Department of Virology,[1] University of Helsinki, and the Department of Viral Biology, Virology Division, USAMRIID, Fort Detrick, the prime goal has been to adapt the causative agent of nephropathia epidemica (NE), the Scandinavian form of Korean hemorrhagic fever (KHF) virus, to cell culture, and thus enable a close characterization of the agent. In addition, some emphasis was put into further characterizing KHF virus, which has been successfully propagated in cell cultures at Fort Detrick.

The report will contain the following parts:

- cell culture work with nephropathia epidemica agent,
- rodent inoculation experiments with NE,
- serological comparison of NE and KHF, and
- KHF characterization attempts

[1]The demonstration of nephropathia epidemica agent in 1978, and its further passaging in voles, has been carried out at the Department of Virology, University of Helsinki, by a research group led by Dr. M. Brummer-Korvenkontio.

1. CELL CULTURE WORK:

The prime objective of this project was to adapt the NE agent to cell cultures in the same way it had been achieved with KHF. For this purpose, the following samples were brought from Finland:

a. Vole lung samples (all of these C. glareolus vole lungs originated from a single isolate, the Sotkamo strain of NE, which had been passaged four times in voles at the Department of Virology, University of Helskinki).

b. Two pools of trypsinized and washed C. glareolus lung cells, each containing cells from three voles. The rationale of these samples was that they would be void of antibodies against NE.

c. Urine from one NE positive vole.

The samples are presented in Table 1.

Although all attempts to adapt the NE agent to cell culture were unsuccessful, it may be of some importance to report all the details of the procedures used. This may be of some help in planning future isolation experiments.

a. Cells. A-549 and Vero E6 cells were used at passage levels not exceeding 30. The cells were grown in T25 flasks and delivered from the cell culture department as subconfluent.

b. Media. E-199 media supplemented with 10% heat-inactivated fetal calf serum and garamycin.

c. Inocula. For the primary inoculations, vole lung pieces of about 0.5 g were homogenized with a tissue homogenizer in 1.5 ml of E-199 media and used as such.

d. Infection. Two modes of infection were used:

(1) Infection of subconfluent monolayers. The flasks were washed once with prewarmed E-199 media and then inoculated with 0.2 ml of lung suspension. After 1 hour's adsorption at 37°C, 6 ml of media was added to the flask without removing the inoculum.

(2) Infection of cells in suspension. For this purpose the cells were trypsinized and washed twice in serum-containing media. After the second wash, the cells were resuspended in 0.7 ml media. Then 0.3 ml of inoculum was added and the cells were then incubated for 45 minutes at 37°C. After adsorption, the cells were transferred to a T25 flask and 6 ml media was added. The cells were then grown for 2 weeks at 37°C in a CO_2 incubator.

e. Growth. The infected cell cultures, as well as the uninfected control cultures, were grown for 2 weeks at 37°C in a CO_2 incubator without media change. The cells were observed by light microscopy two to three times weekly for possible CPE. Both the infected cultures, as well as the cell controls, showed an unaltered cell morphology during the growth period.

f. Passages. Three kinds of samples were generated at the end of each passage:

(1) supernatant,

(2) frozen and thawed cells, and

(3) trypsinized cells for further cultivation.

The samples (1) and (2) were inoculated on new cell cultures (0.2 ml inoculum in a T25 flask). The trypsinized cells were passed in two new

flasks at a split ratio of 1:5. Each sample underwent four successive blind passages.

g. Testing of the samples. At each passage level the cells were controlled for possible NE positivity by immunofluorescence. For this purpose trypsinized cells were seeded on pre-sterilized spot slides at a cell density of 10^5 cells/50 µl and allowed to grow in closed chambers for 12 hours at 37°C in a CO_2 incubator. The medium was then removed and the slides allowed to air dry at room temperature. The cells were then fixed by immersing the slides into ice cold acetone for 5 minutes. The slides were then air dried, stained for immunofluorescence or stored at -70°C until used.

Before staining the cells were washed three times with PBS. Human convalescent NE serum (R.T. 1:20 dilution and T.M. 1:100 dilution in PBS) was used as first antibody. As a second antibody, FITC-conjugated goat anti-human IgG (Miles - Yeda) was used. After both serum incubations the samples were washed thoroughly with PBS, and finally with distilled water, before mounting them with glycerol - PBS (9:1).

No FA positivity was found in any of the tested NE samples. KHF spot slides, which were used as a positive control, showed a typical punctate cytoplasmic fluorescence in every instance.

2. RODENT INOCULATIONS:

In order to try to establish the Sotkamo strain of NE at Fort Detrick, some of the lung samples were inoculated into colonized voles. These voles were all from the same colony of North American C. gaperi voles kept at Fort Detrick for more than a year.

a. Inoculation. Lung suspensions, made as described above, were used as inocula. A 0.1 ml aliquot of the suspension was injected directly into the lung through the thoracic wall. Ten to 15% of the voles died within 24 hours after inoculation, apparently due to injuries caused by the injection. Each sample was inoculated into five voles. The animals were sacrificed 1 to 4 weeks after inoculation. The following samples were collected: a blood sample by heart puncture, the lungs, as well as samples of liver, kidney, and spleen. The serum was separated from the blood samples for antibody determinations. The organs were stored at -70°C until tested for presence of antigen.

b. Testing of samples.

(1) Serum. The possible presence of antibodies against NE was tested using KHF spot slides (Vero or A-549 cells infected with Hanvirus - a strain of KHF). The vole sera were diluted in 4-fold dilutions in PBS, starting with a 1:20 dilution. FITC-conjugated goat anti-mouse IgG was used as second antibody. This conjugate appeared to be rather sensitive in detecting vole Ig, although we do not know to which degree the obtained titers are reduced. The same conjugate also gave comparable titers of rat anti-KHF antibodies, as compared to the homologous system. The titer in the vole sera was determined within the accuracy of two-fold dilutions by estimating the strength of the fluorescence in the last positive dilution.

(2) Organs. The lungs were primarily used to test for possible presence of NE antigen in the voles. The frozen tissue was embedded in OCT compound on a cryostat holder, brought into the cold chamber of the cryomicrotome and allowed to stay there for about 15 minutes in order to equilibrate the temperature to the -20°C used. Sections of 4 µm were cut

and two sections were picked up on each microscope slide. The sections were air-dried and either stained immediately or stored at -70°C until used.

c. FA staining. Essentially the same procedure as described for the spot slide staining was used, with the exception that the washings after each antibody incubation was intensified. A 50 µl drop of the diluted serum was applied on each section. The samples were observed in a Leitz fluorescence microscope using a 40 X oil immersion lens. The result was scored from negative to ++++ by estimating the strength of positivity. In all cases the positive fluorescence occurred as a granular cytoplasmic material (vacuoles?). The strongest positive samples showed fluorescence in essentially all cells, whereas the weakly positive samples only contained focally positive cells.

d. Findings. In the first experiment, a selection of the Finnish vole lung samples was injected into five C. gaperi voles each. The results are summarized in Table 2. It shows that four out of eight tested samples were infectious, and that in all instances both antibodies and antigen was demonstrable in the infected animals. The results strongly suggest that vole V2960, one of the three voles collected from nature and from which the Sotkamo strain of NE originates, would be the origin of the strain. Vole V3036 appears to be a good source for further experiments. It is of interest to note that no antibodies could be demonstrated in the serum of this vole.

Table 3 summarizes an attempt to titrate the amount of infectivity in the lung sample of V2969. It shows a rather anomalous titration behavior without an apparent dilution dependence. The result

may at least partly be due to the fact that the inoculum is rather inhomogenous, containing actually also small pieces of lung tissue. In the next experiment we tested whether the infectivity of NE was retained in C. gaperi. For this experiment we selected two vole lung samples from the first passage: V2969/1, which contained lots of antigen, but a moderate antibody titer; and V3036/4, with moderate antigen content, but high antibody titer. As shown in Table 4, both of these samples contained infectivity. It thus appears that the NE agent can be successfully passaged in the C. gaperi voles. This is the only other rodent, apart from C. glareolus, where NE has been found to propagate.

Lastly, we wanted to test if NE agent would be secreted into the urine of infected voles, as has been shown to be the case of KHF in Apodemus (by Lee and co-workers). Urine, being void of antibodies, would appear as a good source for cell culture adaptation experiments. Fifteen (15) voles were injected with V3036 lung suspension. At 1 week intervals, six voles were placed separately into metabolic cages and urine collected for 3 to 5 hours. The urine was collected into small beakers with 1 ml of EMEM (5% fetal calf serum, 10 mM Hepes, garamycin). Two to 4 ml of urine was obtained during the collecting period. Three of the voles were subsequently sacrificed and tested for possible presence of antibodies and antigen in lungs. The other three voles were saved for later sampling. Four weeks after inoculation, the remaining six voles were sacrificed after urine collection.

Urine samples, from the above voles of week 1, 2, and 3, were inoculated into vole lungs immediately after collection. Each urine sample was inoculated into three voles. In these experiments the initial

death rate (within 24 hours) was very high (i.e., 9/27). We do not know the reason for this, but it may be due to the urine samples often showing contamination by bacteria.

The results of this experiment are summarized in Table 5. It shows, firstly, that all voles injected became infected, indicating a very good infectivity in V3606. It can also be seen that after 1 week antigen can be detected in the lungs, but no antibodies are yet detected in serum. Two weeks after inoculation, both antigen and antibodies are present. The results clearly indicate that urine of infected voles is infectious. The results point out that NE agent is secreted into urine very early during the infection, although the low number of surviving animals does not allow any strong conclusions in this respect.

It will be of great importance to quantitate the amount of infectivity in the urine before the value of this material for cell culture isolation attempts can be evaluated. Even without this knowledge, initial experiments with inoculations of cell cultures with urine were started. These were, however, unsuccessful, due to bacterial contamination.

3. SEROLOGY:

Initial experiments were carried out in order to characterize the antigenic relationship between KHF and NE. For these experiments, two sets of serum samples were available: a panel of 24 sera (15 human and 9 rodent sera) from different parts of the world and positive for KHF or NE, and a panel of sera from 28 Finnish NE patients and NE negative controls with 19 paired or multiple serum samples.

The first panel was tested by immunofluorescence using NE-infected vole lung sections as antigen. Out of these sera, the only positive one was a Scandinavian NE convalescent serum.

The second panel had been previously screened with NE antigen (M. Brummer-Korvenkontio and A. Vaheri, Department of Virology, University of Helskini). These sera were now tested by immunofluorescence using KHF spot slides as antigen. These results have not been completed, but the following conclusions can already be made:

a. KHF antigen measures NE antibodies very efficiently; none of the sera found positive by NE antigen was missed when using KHF antigen. The titers were also relatively high and compared well with the ones obtained with NE antigen.

b. In several instances paired sera, which when tested with NE antigen showed a seroconversion, showed a moderate titer in the acute phase sample by KHF spot slides. It is not clear why the spot slide system seems to be more sensitive when measuring antibodies of the early phase of the disease. It is tempting to suggest that this may be due to the ability of this system to detect antibodies of the IgM class. Repeated experiments in Helsinki have, however, failed to demonstrate any antibodies of this class (A. Vaheri and M. Brummer-Korvenkontio, unpublished).

The serology data clearly demonstrates that there is a very strong one-way cross-reaction between KHF and NE. More sensitive measurements, using RIA and ELISA techniques, were initiated to determine the degree of cross-reactivity between these two agents more accurately.

In a very limited study in collaboration with Dr. J. LeDuc, Medical Division, USAMRIID, it could be established that in 2 out of 15 rat sera collected from rural Southern Finland, antibodies detectable with KHF spot slides were present. The type of antigen behind these rats cannot at present be established, since the rat sera cannot reliably be tested with the vole lung antigen.

4. ATTEMPTS TO CHARACTERIZE KHF:

One of the goals was to obtain more information about the structure and morphogenesis of KHF virus. Earlier work at Fort Detrick indicated a Bunyavirus-like structure for this agent.

In collaboration with Dr. J. White, USAMRIID, and others, attempts were made to grow radioactively labeled KHF virus (^{3}H-uridine and ^{14}C-amino acids). It became apparent, however, that the production of virus in the A-549 cells was not sufficient to allow its detection by radioactivity. In other experiments, KHF was grown in A-549 cells, the supernants collected, and cell debris removed by low-speed centrifugation. In a single ultracentrifugation step, a 50- to 100-fold concentrated sample was recovered from the interface, 15 and 50% sucrose. In this sample, particles compatible with Bunyavirus structure could be seen by negative staining. These particles could be enriched on the electron microscope grids if these were coated with KHF antibodies prior to applying the virus onto the grid. The amount of virus in these samples was now, however, sufficient to allow any detailed structural studies. In none of the numerous samples of the infected cell cultures processed for thin sectioning could any particles of viral appearance be found, nor

could any changes in the cell morphology, as compared to the control cells, be detected.

It is clear that more emphasis has be be put into optimizing the growth conditions of the KHF virus in cell cultures before a reliable structural analysis of the virus and its morphogenesis is possible.

5. CONCLUSIONS:

The causative agent of nephropathia epidemica, the Scandinavian form of KHF, has been studied in passaging experiments in rodents and in cell culture. The following findings were made:

a. The NE agent (Sotkamo strain) from Finnish C. glareolus vole lungs could be propagated in colonized C. gaperi voles.

b. The agent is demonstrable in injected voles already 1 week after inoculation. At this time antibodies against NE were not detectable in the blood of the infected animals.

c. In voles injected with NE, the agent is secreted into the urine. The agent is demonstrable already 1 week after inoculation (i.e., at the same time it appears in the lungs).

d. All attempts to isolate the NE agent in cell culture were unsuccessful.

This work has provided knowledge and material for further isolation attempts. These, as well as further characterization of the serological relationships between NE and KHF, are in progress. The collaboration between the Virology Division, USAMRIID, Fort Detrick, and the Department of Virology, University of Helsinki, will thus hopefully be continued.

6. GENERAL REMARKS:

The collaboration and help at USAMRIID, especially by Dr. Joel Dalrymple, but also by the whole staff at the Virology Division, as well as at the Electron Microscopy Department, has been of the best quality and certainly forwarded the probject in every possible way. The standard and performance of the laboratories has been a pleasure to enjoy. A few suggestions for further improvement of the laboratories may be allowed:

a. It would appear practical to have access to a "cold" laboratory in connection with the "hot" ones. Much of the virus work does not require stringent isolation conditions and would be more practical to perform in a "cold" area.

b. It would be helpful (especially for newcomers) if some kind of service connection between the "hot" laboratories and the outside would exist. At present, all forgotten items lead to an extra shower.

c. A minor source of irritation was the supply of items in the locker room. Working in a green suit, small model, when being of my size, large, or using your shirt as a towel after the shower, may cause stress.

It must be pointed out that the criticisms given here by no means shadows the overall positive impression of the laboratory.

TABLE 1.

Voles collected from nature C. glareolus from Sotkamo (sample)	Amount of antigen in lungs	Antibody titer
V2967 (lung)	±	160
V2969 "	++	640
V2971 "	++	160
Voles injected with pool of above vole lung suspension		
V3036 (lung)	+++	<20
V3046 "	+++	40
Voles injected with V3036 lung suspension		
V3117 (lung)	+	ND
V3135 "	++	ND
V3115 "	+++	ND
V3115 (urine)		
Pool I (trypsinized lung cells from 3 voles)		
Pool II (- " -)		

TABLE 2

Inoculum	No voles inoculated/ no NE positive	NE positive voles		
		No	Amount of Ag in lungs	Ab titer
V2967	4 / 0			
V2969	4 / 3	1	++++	80
		2	++++	80
		3	+++	80
V2971	3 / 0			
V3036	4 / 4	1	+	160
		2	+++	640
		3	+++	80
		4	++	320
V3046	4 / 2	1	+++	160
		2	+++	40
V3117	4 / 1	1	+++	160
Pool I	3 / 0			
Pool II	3 / 0			

1) All positive voles showed both antigen and antibodies against NE. the rest of the voles had antibody-titers of 20 and no antigen demonstrable in lung sections

TABLE 3.

Dilution of inoculum (V2969 lung suspension)	Vole no	Amount of Ag in lung	Ab titer
10^0	1	-	<20
	2	+++	160
	3	+++	80*
	4	-	<20
10^1	1	+++	80
	2	-	<20
	3	+++	320
	4	-	<20
	5	-	<20
10^2	1	-	<20
	2	-	<20
	3	-	<20
	4	-	<20
10^3	1	-	<20
	2	-	<20
	3	+++	320
	4	-	<20
10^4	1	-	<20
10^5	1	-	<20
	2	-	<20
	3	-	<20
	4	-	<20
	5	-	<20

TABLE 4.

Inocula[1]	vole no	Amount of Ag in lung	Ab titer
V2969/1 lung susp.	1	+	80
	2	+	320
	3	+	80
	4	+++	320
	5	-	320
V3036/4 lung susp.	1	-	ND
	2	+	320
	3	++	320
	4	-	

1) Identification of sample as in Table 2

TABLE 5.

Voles injected with V3036 lung suspension				Urine inoculated voles[1]		
Week post inocul.	Vole No	Amount of Ag in lung	Ab titer	Vole No	Amount of Ag in lung	Ab titer
1	1	+	<20	1	+++	160
				2	-	<20
				3	-	<20
	2	++	<20	1	-	<20
	3	+++	<20	1	-	<20
				2	-	<20
2	1	+	40	1	+++	40
	2	++	80	1	++++	80
	3	+++	160	1	-	<20
				2	-	<20
3	1	+	<20	1	-	<20
				2	-	<20
	2	++	<20	1	-	<20
				2	-	<20
				3	-	<20
	3	+++	320	1	-	<20
				2	-	40
4	1	++	160	Urine from these animals was		
	2	++	160	not inoculated in further voles		
	3	+	320			
	4	ND	320			
	5	+++	40			
	6	+++	160			

1) these voles were sacrificed 2-3 weeks after inoculation

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